Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy

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Cardiac hypertrophy is a major cause of morbidity and mortality worldwide. The hypertrophic process is mediated, in part, by small G proteins of the Rho family. We hypothesized that statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, inhibit cardiac hypertrophy by blocking Rho isoprenylation. We treated neonatal rat cardiac myocytes with angiotensin II (AngII) with and without simvastatin (Sim) and found that Sim decreased AngII-induced protein content, [^{3}H] leucine uptake, and atrial natriuretic factor (*ANF*) promoter activity. These effects were associated with decreases in cell size, membrane Rho activity, superoxide anion ($O_2 \cdot ^-$) production, and intracellular oxidation, and were reversed with L-mevalonate or geranylgeranylpyrophosphate, but not with farnesylpyrophosphate or cholesterol. Treatments with the Rho inhibitor C3 exotoxin and with cell-permeable superoxide dismutase also decreased AngII-induced $O_2 \cdot ^-$ production and myocyte hypertrophy. Overexpression of the dominant-negative Rho mutant N17Rac1 completely inhibited AngII-induced intracellular oxidation and *ANF* promoter activity, while N19RhoA partially inhibited it, and N17Cdc42 had no effect. Indeed, Sim inhibited cardiac hypertrophy and decreased myocardial Rac1 activity and $O_2 \cdot ^-$ production in rats treated with AngII infusion or subjected to transaortic constriction. These findings suggest that statins prevent the development of cardiac hypertrophy through an antioxidant mechanism involving inhibition of Rac1.

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Introduction

Cardiac hypertrophy represents an initial physiological adaptive response to increases in blood pressure or afterload (1). However, despite normalization of systemic blood pressure by medication, cardiac hypertrophy frequently decompensates into congestive heart failure (2). Indeed, cardiac hypertrophy is an independent risk factor in cardiovascular disease, and increases cardiovascular mortality by more than twofold (3). The hypertrophic process is characterized by the induction of immediate-early genes such as *c-fos*, *c-jun*, and *erg-1*; the reexpression of embryonic genes such as atrial natriuretic factor (ANF), β-myosin heavy chain, and skeletal α-actin (4); and the increased synthesis of contractile proteins such as myosin light chain-2 (MLC-2v) and cardiac α-actin (5, 6).

Stimulation of the angiotensin II (AngII) type I receptor or exposure to pressure overload induces a cardiac hypertrophic response mediated in part by the activation of the heterotrimeric G proteins (e.g., G_q) (7, 8) and small G proteins (e.g., Ras and Rho) (9–11). In particular, the Rho proteins, RhoA, Rac1, and Cdc42, are thought to play critical roles in the hypertrophic process by regulating cell morphology and contractile elements

(12). For example, previous studies have shown that RhoA is required for $G\alpha_q$ and α_1-receptor signaling in cardiac myocytes, and that dominant-negative mutants of RhoA attenuate cardiac myocyte hypertrophy in response to stimulation with phenylephrine (11, 13). Surprisingly, cardiac-specific overexpression of RhoA in mice does not lead to cardiac hypertrophy, but produces sinus and atrioventricular nodal dysfunction and development of ventricular failure (14). These results suggest that other pathways may contribute to the hypertrophic response. Indeed, Rac1, and not RhoA, is required for signal transduction pathways leading to cardiac myocyte hypertrophy (15).

Current treatments for cardiac hypertrophy are limited to vasodilators or afterload reducers, with few if any therapies directed at the myocardial process. The 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors, or statins, are widely prescribed cholesterol-lowering agents that decrease the incidence of myocardial infarction and ischemic stroke (16, 17). In addition to inhibiting cholesterol synthesis, the statins also inhibit the synthesis of important isoprenoid intermediates that are important lipid attachments required for the subcellular localization and function

of a variety of proteins, including the Rho proteins (18, 19). The purpose of this study, therefore, was to determine whether statins can attenuate cardiac hypertrophy by inhibiting Rho proteins, and if so, to determine the potential underlying mechanism involved.

Methods

Cardiac myocyte culture. We obtained ventricles from 1-day-old Sprague-Dawley rats, and isolated cardiac myocytes by digestion with trypsin-EDTA and type 2 collagenase as described (20). Cells were cultured in serum-free insulin-transferrin (IT) medium (21) for an additional 24–36 hours. Using this method, we obtained primary cultures of greater than 95% cardiac myocytes, as assessed by microscopic observation of spontaneous cellular contractions and immunofluorescence staining of anti-cardiac myosin heavy chain. Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), and were used at the following final concentrations: AngII, 1 μM; preactivated simvastatin (Sim; Brigham & Women's Hospital Pharmacy), 0.1–10 μM; L-mevalonate (L-mev), 200 μM; farnesylpyrophosphate (FPP), 10 μM; geranylgeranylpyrophosphate (GGPP), 10 μM; LDL cholesterol, 1 mg/ml; farnesyltransferase inhibitor (FTI-276; Calbiochem-Novabiochem Corp., San Diego, California, USA), 20 nM; geranylgeranyl transferase inhibitor (GGTI-286; Calbiochem-Novabiochem Corp.), 30 μM; *Clostridium botulinum* C3 transferase (C3 TF; List Biological Laboratories Inc., Campbell, California, USA), 50 μg/ml; polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), 50 U/ml; polyethylene glycol–conjugated catalase (PEG-CAT), 500 U/ml; and polyethylene glycol (PEG), 0.32 mg/ml. The PEG, PEG-SOD, and PEG-CAT were dissolved in IT medium and incubated with cardiac myocytes for 1 hour before AngII stimulation. Cardiac myocytes were stimulated for 24 hours. Viability was determined by cell number, frequency of contractions (i.e., intrinsic heart rate), cellular morphology, and trypan blue exclusion.

Measurements of cardiac hypertrophy. We measured [³H]leucine uptake as described (22). Cardiac myocytes were incubated for 36 hours in serum-free IT medium, then treated under the indicated conditions in the presence of diluent or [³H]leucine (1 μCi/ml) for 24 hours. After incubating at room temperature for 45 minutes, cellular proteins were precipitated with 5% trichloroacetic acid and resuspended in 0.4 N NaOH, and the radioactivity was counted in a scintillation counter (Beckman LS 6000IC; Beckman Instruments Inc., Fullerton, California, USA).

Northern blotting. Equal aliquots of total RNA (15 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as previously described (23). The ANF and MLC-2v cDNAs were labeled with random hexamer primer using [α-³²P]CTP (NEN Life Science Products Inc., Boston, Massachusetts, USA) and Klenow (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey,

USA). Loading conditions were determined by ethidium bromide staining of 28S ribosomal RNA. Blots were analyzed by laser densitometry.

Transient transfections. Nearly confluent rat cardiac myocytes were transfected with the indicated cDNAs using the calcium-phosphate coprecipitation method (24). The following amounts of cDNAs were used: mutant c-myc-tagged RhoA, Rac1, and Cdc42 (10 μg each); ANF-Luc reporter plasmid (15 μg); and β-galactosidase plasmid (4 μg). Approximately 48 hours after transfection, the myocytes were treated as indicated for an additional 24 hours. Luciferase activity was normalized to β-galactosidase activity for each sample, to correct for variations in transfection efficiency. Results are expressed as relative ratio of control (fold induction). Preliminary studies indicate that the transfection efficiency was approximately 2–4%.

Immunofluorescence. Following transfection and stimulation under the indicated conditions, rat cardiac myocytes were treated with 10 μg/ml of dichlorofluorescin-diacetate (Molecular Probes Inc., Eugene, Oregon, USA) for 1 minute, then fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100. For staining of the c-myc tag, cells were incubated with mouse anti-human c-myc antibody (1:100 in 1% BSA; Transduction Laboratories, Lexington, Kentucky, USA) at 37°C for 20 minutes. After cells were washed with 1% BSA, R-phycoerythrin–conjugated goat ant-mouse IgG (red fluorescence) was used as secondary antibody (1:100 in 1% BSA). For staining of α-actinin and desmin, cells were incubated with a mouse monoclonal antibody to α-actinin (1:200 in 1% BSA) and a rabbit polyclonal antibody to desmin (1:100 in 1% BSA; Sigma Chemical Co.). After cells were washed with 1% BSA in PBS, they were treated with these secondary antibodies: FITC-conjugated goat anti-mouse IgG (H+L) (green fluorescence) and TRITC-conjugated anti-rabbit IgG (H+L) (red fluorescence) (1:200 in 1% BSA; Molecular Probes Inc.). Immunofluorescence was visualized using an MRC-1024/2P multiphoton microscope equipped with krypton-argon and Ti-sapphire lasers (Bio-Rad Laboratories, Hercules, California, USA). Photographic images were taken from five random fields.

Rho GTP-binding activity. We measured membrane-associated Rho GTP-binding activity in cultured cardiac myocytes or rat hearts by immunoprecipitating [³⁵S]GTP-γS-labeled Rho proteins using partially purified cardiac myocyte membranes and specific RhoA and Rac1 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) as described (24). Immunoblotting of leftover supernatants indicated that RhoA and Rac1 were completely immunoprecipitated. Nonspecific activity was determined in the presence of excess unlabeled GTP-γS (100 μM; NEN Life Science Products Inc.).

Ferricytochrome c reduction assay. Assay for superoxide anion ($O_2 \cdot^-$) released into the supernatant was carried out by measuring superoxide dismutase–inhibitable (SOD-inhibitable) reduction of ferricytochrome *c* as

described (25). After 24 hours of stimulation in phenol red-free and serum-deficient IT medium, ferricytochrome c was added to the supernatant to a final concentration of 70 μM in the presence or absence of SOD (100 U/ml). Reduction of ferricytochrome c in the supernatant was monitored for 10 minutes at an absorbance of 550 nm using a spectrophotometer (SPECTRAmax PLUS[384]; Molecular Devices Corp., Sunnyvale, California, USA). Rates of $O_2.^-$ production were calculated as described (26). The results are expressed as nmol/24 h/million cells.

Measurement of $O_2.^-$ production from heart tissues. Intact, nonhomogenized pieces of rat or mouse hearts (100–200 mg) were suspended in a Krebs bicarbonate buffer containing the following reagents, in mmol/l: NaCl, 118; KCl, 4.7; $CaCl_2$, 1.5; $MgSO_4$, 1.1; KH_2PO_4, 1.2; glucose, 5.6; and $NaHCO_3$, 25. This tissue bath was adjusted to pH 7.4, and gassed with 21% O_2 and 5% CO_2 for 4 hours. The resulting supernatant was used for measuring SOD-inhibitable reduction of ferricytochrome c as described (25). The results were expressed as nmol of $O_2.^-$ produced per gram of cardiac tissue.

Aconitase activity assay. The production of intracellular $O_2.^-$ was determined indirectly by changes in aconitase activity (27). Cultured cells were solubilized in a lysis buffer containing PBS, Triton X-100 (0.2%), DTPA (100 mM), and citrate (5 mM). Heart tissues were homogenized and resuspended in a buffer containing Tris-HCl (50 mM, pH 7.6), cysteine (1 mM), citrate (1 mM), and $MnCl_2$ (0.5 mM). Cellular extracts (15 μg of protein) were added to a reaction buffer (0.2 ml) containing Tris-HCl (50 mM, pH 7.4), isocitrate (20 mM), and $MnCl_2$ (0.5 mM) at 25°C, and the formation of *cis*-aconitate from isocitrate was measured spectrophotometrically after 2 minutes, at an absorbance of 240 nm. Aconitase activity was calculated using the extinction coefficient of 3.6 mM^{-1} × cm^{-1} and expressed as nmol of *cis*-aconitate converted/min/mg protein (28).

Animal models of cardiac hypertrophy. We used male Sprague-Dawley rats (8 weeks old, 200–250 g) from Taconic Farms (Germantown, New York, USA). Rats received saline (25 μl/h) or AngII (200 ng/kg/min) by osmotic minipumps (ALZET 2002; ALZA Corp., Palo Alto, California, USA), with and without 1.0 ml of pre-activated Sim (0.2, 2, or 20 mg/kg/day, subcutaneous) or a corresponding volume of PBS for 14 days. Systolic blood pressure was measured noninvasively using the tail-cuff method (IITC Inc., Woodland Hills, California, USA). Left ventricular (LV) mass, body weight (BW), and serum total cholesterol level were determined for each condition before and after treatment.

For the pressure-overload model, we used male C57BL/6 mice (8 weeks old, 20–25 g) from Charles River Laboratories (Wilmington, Massachusetts, USA). Transaortic constriction (TAC) was created using a 7-0 suture tied twice around the aorta and a 27-gauge needle. The needle was then gently retracted, yielding a 60–80% constriction with an outer aortic diameter of approximately 0.3 mm. Mice received either vehicle or

preactivated Sim (2 mg/kg/day, subcutaneous) immediately after surgery. After 4 weeks of TAC, mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and cardiac dimensions and function were analyzed by 15-MHz pulse-wave Doppler echocardiography (SONOS 5500; Hewlett-Packard, Andover, Massachusetts, USA). Systemic arterial blood pressure was measured invasively via the left carotid artery.

Results
Cell culture. Relatively pure (>95%) neonatal ventricular myocytes were confirmed by their morphological features using phase-contact microscopy and immunofluorescent staining with a monoclonal anti-cardiac MHC antibody (data not shown). There were no observable adverse effects by AngII, Sim, L-mev, LDL, GGPP, FPP, GGTI-286, FTI-276, C3 TF, PEG-SOD, or PEG-CAT on cellular viability for any treatment conditions.

Statins inhibit AngII-induced cardiac myocyte hypertrophy. Cardiac myocyte hypertrophy is characterized by increased protein content (e.g., [3H]leucine uptake) and induction of fetal gene expression (e.g., *ANF* promoter activity). Treatment with AngII induced neonatal rat cardiac myocyte hypertrophy, as measured by changes in [3H]leucine uptake, total protein content, and *ANF* promoter activity (50% ± 6%, 20% ± 3%, and 180 ± 20%-fold increases, respectively; $P < 0.01$ for all conditions) (Figure 1a). Cotreatment with Sim (5 μM) inhibited the AngII-induced increases in [3H]leucine incorporation, total protein content, and *ANF* promoter activity by 71% ± 4%, 85% ± 8%, and 94% ± 5%, respectively. These inhibitory effects of Sim were completely reversed by cotreatment with L-mev or GGPP, but not with FPP or LDL (Figure 1). Treatment with Sim alone, however, did not affect basal [3H]leucine incorporation, total protein content, or *ANF* promoter activity. Similar effects were observed with other statins (e.g., lovastatin, atorvastatin), indicating a class effect of these agents on myocardial HMG CoA reductase.

Using selective inhibitors of geranylgeranylation and farnesylation at their optimal inhibitory concentrations, we found that GGTI-286 (30 μM), but not FTI-276 (20 nM), completely blocked AngII-induced [3H]leucine incorporation (Figure 1b). To determine whether inhibition of Rho proteins could be mediating the effects of Sim on cardiac myocyte hypertrophy, Rho proteins were directly inhibited by C3 TF (50 μg/ml) (29). Treatment with C3 TF inhibited the AngII-induced increase in [3H]leucine incorporation by 59% ± 5%. In contrast, direct activation of Rho by *Escherichia coli* cytotoxic necrotizing factor-1 (30) partially reversed the inhibitory effects of Sim (data not shown). Since the Rho protein Rac1 is involved in the generation of reactive oxygen species (31, 32) and mediates signaling pathways leading to cardiac hypertrophy (15), we investigated whether superoxide anion ($O_2.^-$) or hydrogen peroxide (H_2O_2) could be playing a role in AngII-induced cardiac hypertrophy. Cotreatment with cell-permeable
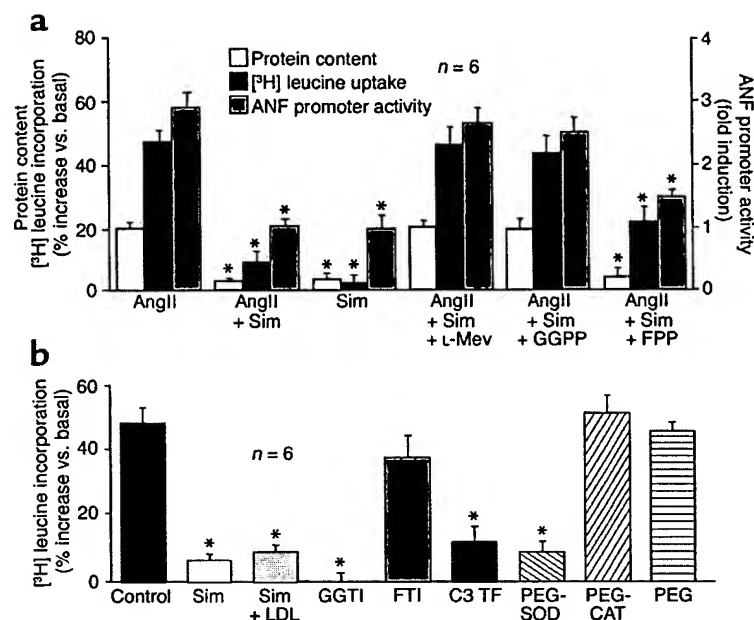
Figure 1

Inhibition of cardiac myocyte hypertrophy by statins. (a) Effects of AngII or Sim (5 μM) with and without L-mev, GGPP, or FPP on total protein content, [³H] leucine incorporation, and ANF promoter activity. Values are expressed as mean ± SEM. *P < 0.01 compared with AngII alone. (b) Effects of Sim (5 μM) with and without LDL, GGTI, FTI, C3 TF, PEG, PEG-SOD, and PEG-CAT on AngII-induced [³H] leucine incorporation (Control). Values are expressed as mean ± SEM. *P < 0.01 compared with control.
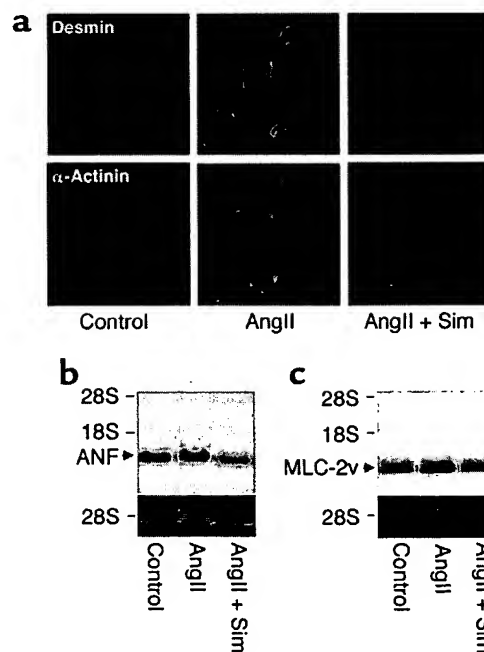
PEG-SOD, but not PEG-CAT or PEG alone, inhibited AngII-induced [³H]leucine incorporation (Figure 1b). These findings suggest that the hypertrophic process is more dependent upon $O_2{}^{.-}$ than on H_2O_2.

To determine whether Sim can affect cardiac myocyte cell size and sarcomeric organization, we immunostained cardiac myocytes for two sarcomere-associated proteins, desmin and α-actinin. Treatment with AngII induced significant increases in cell size and sarcomere organization (Figure 2a); cotreatment with Sim blocked these AngII-induced cellular changes. The hypertrophic process is also associated with the induction of fetal and structural cardiac genes such as ANF and MLC-2v (4, 33). Indeed, steady-state ANF and MLC-2v mRNA expression were each increased by about twofold following AngII stimulation (n = 3, P < 0.01) (Figure 2, b and c). Cotreatment with Sim inhibited AngII-induced ANF, and to a lesser extent, MLC-2v mRNA expression

Figure 2

Inhibition of cardiac sarcomere organization, myocyte size, and fetal gene expression by statins. (a) Effects of AngII with and without Sim (5 μM) on cardiac myocyte size and sarcomere organization. Double immunofluorescent microscopy was performed using specific antibodies to desmin (upper panel, red color) and α-actinin (lower panel, green color). Experiments were performed three times with similar results. Effect of AngII with and without Sim (5 μM) on steady-state (b) ANF and (c) MLC-2v mRNA expression after 24 hours of treatment. Corresponding ethidium bromide–stained 28S ribosomal RNA was used to standardize loading. The results shown are representative of three separate experiments.
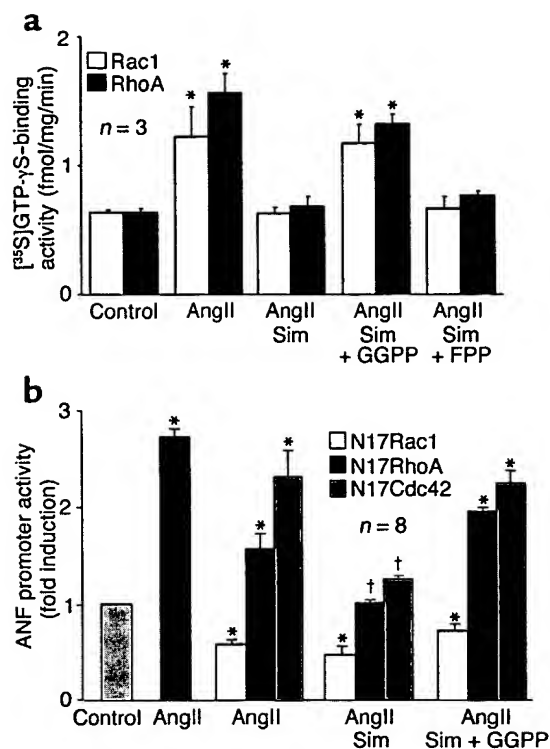
in cardiac myocytes. High basal expression of ANF and MLC-2v is attributed to the use of neonatal cardiac myocytes instead of adult cardiac cells. Nevertheless, these findings correlate with the inhibition of cardiac myocyte hypertrophy by Sim.

Inhibition of cardiac hypertrophy by statins is mediated by Rac1 and RhoA. Treatment with AngII increased membrane-associated RhoA and Rac1 GTP-binding activity by 147% and 87%, respectively (P < 0.05 for both) (Figure 3a). Cotreatment with Sim completely blocked AngII-induced increases in membrane-associated RhoA and Rac1 GTP-binding activity. These inhibitory effects of Sim were reversed by cotreatment with GGPP, but not FPP, indicating that Sim inhibited the membrane localization and activities of both RhoA and Rac1.

To assess which Rho protein mediates AngII-induced cardiac hypertrophy, we cotransfected rat cardiac myocytes with dominant-negative mutants of Rac1, RhoA, or Cdc42 and an ANF promoter–luciferase reporter construct. Transfection with dominant-negative Rac1 (N17Rac1), and to a lesser extent, RhoA (N19RhoA), inhibited AngII-induced ANF promoter activity (Figure 3b). Transfection with Cdc42 (N17Cdc42) had no inhibitory effect. Cotreatment with Sim further decreased ANF promoter activity in cells transfected with N19RhoA and N17Cdc42, but not N17Rac1. Indeed, N17Rac1 decreased ANF promoter activity to below basal levels (P < 0.05). Although cotreatment with GGPP reversed the inhibitory effects of Sim,
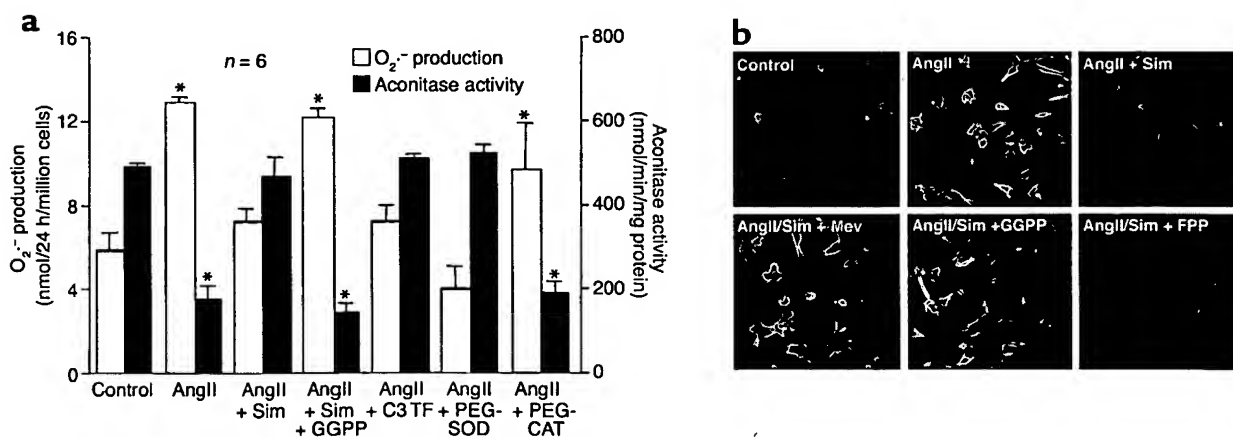
Figure 3
Inhibition of RhoA and Rac1 by statins. (a) Effect of AngII with and without Sim, GGPP, or FPP on membrane-associated Rac1 and RhoA GTP-binding activity in rat cardiac myocytes. Values are expressed as mean ± SEM. *$P < 0.05$ compared with unstimulated cells (Control). (b) Effects of transfection with dominant-negative Rho mutants (N17Rac1, N19RhoA, or N17Cdc42) with and without Sim (5 μM) or GGPP on AngII-induced *ANF* promoter activity. Values are expressed as mean ± SEM. *$P < 0.01$ compared with transfection with vector alone (Control). †$P < 0.05$ compared with AngII and dominant-negative Rho alone. Tx: DN-Rho = N17Rac1, N19RhoA, or N17Cdc42.

myocytes using a ferricytochrome c reduction assay (25). AngII doubled $O_2 \cdot^-$ production (Figure 4a). This increase was blocked by cotreatment with Sim, C3 TF, and PEG-SOD (50 U/ml), but not with PEG-CAT (500 U/ml). Treatment with PEG or Sim alone had little or no effect on $O_2 \cdot^-$ production. Cotreatment with GGPP completely reversed the inhibitory effect of Sim on $O_2 \cdot^-$ production, but not the effects of C3 TF or PEG-SOD (data not shown).

To confirm that the effects of AngII and Sim on $O_2 \cdot^-$ production correspond to changes in cellular oxidative stress, we assessed total intracellular oxidation in rat cardiac myocytes by DCF fluorescence. Since H_2O_2 is the downstream dismutated product of $O_2 \cdot^-$, the level of DCF fluorescence, which correlates with increased levels of peroxide-derived reactive intermediates, may also serve as an useful marker for $O_2 \cdot^-$ production. Indeed, AngII-induced DCF fluorescence was completely blocked by the addition of PEG-CAT, which converts H_2O_2 to H_2O, but not by PEG-SOD, which enhances rather than inhibits the conversion of $O_2 \cdot^-$ to H_2O_2 (data not shown). Stimulation with AngII produced a 2.7-fold increase in intracellular oxidative stress, as measured by DCF fluorescence ($n = 3$, $P < 0.05$) (Figure 4b). This increase was completely

GGPP could not reverse the inhibitory effect of N17Rac1 on *ANF* promoter activity. These results suggest that Rac1 is the predominant Rho protein that mediates AngII-induced cardiac hypertrophy.

Statins inhibit Rac1-induced $O_2 \cdot^-$ production. An important function of Rac in neutrophils (34), fibroblasts (31), and vascular smooth muscle cells (32) is facilitating the assembly of NADPH oxidase, which is a major source of $O_2 \cdot^-$ production in these cells. To determine whether the inhibitory effects of Sim on cardiac hypertrophy involve inhibition of $O_2 \cdot^-$ production, we monitored $O_2 \cdot^-$ released into the supernatant by cardiac

Figure 4
Inhibition of superoxide anion production and intracellular oxidation by statins. (a) Effects of Sim (5 μM), GGPP, C3 TF, PEG-SOD, or PEG-CAT on AngII-induced $O_2 \cdot^-$ production in rat cardiac myocytes, as measured by ferricytochrome c reduction and aconitase assays. Values are expressed as mean ± SEM. *$P < 0.01$ compared with unstimulated cells (Control). (b) Effects of Sim (5 μM) with and without L-mev, GGPP, or FPP on AngII-induced intracellular oxidation (DCF fluorescence). Results shown were chosen from five random fields and are representative of three separate experiments.
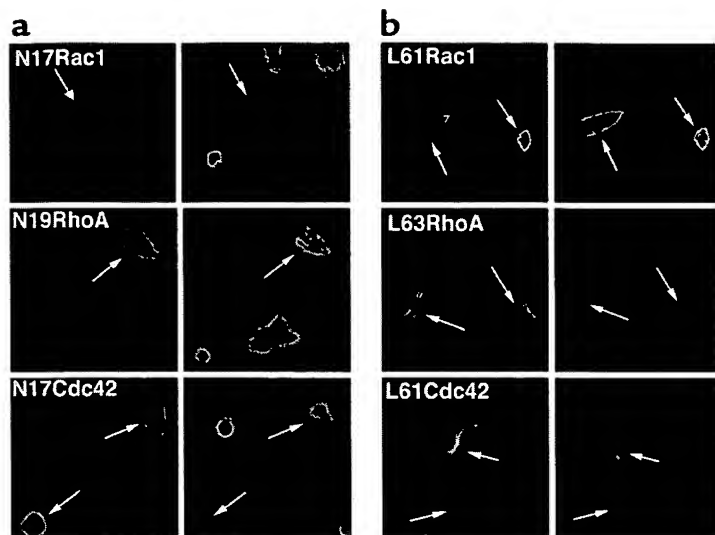
Figure 5

Inhibition of Rac1-induced intracellular oxidation by statins. Intracellular oxidation in rat cardiac myocytes that were transfected with dominant-negative Rho mutants (N17 or N19) and stimulated with AngII (a), or transfected with constitutively active Rho mutants (L61 or L63) (b). Double fluorescent microscopy was performed to determine the expression of c-myc-tagged Rho mutants (left panel, red color) and corresponding levels of DCF fluorescence (right panel, green color). Experiments were performed three times with similar results. The arrows indicate transfected cardiac myocytes in corresponding panels.

blocked by cotreatment with Sim. The inhibitory effect of Sim on intracellular oxidation was reversed by L-mev and GGPP, but not by FPP, suggesting that Rho proteins were mediating the increase in $O_2\cdot^-$-derived intracellular oxidation in response to AngII.

Rac1 is required for AngII-induced intracellular oxidation. To determine which Rho proteins mediate the AngII-induced increase in intracellular oxidation, we transfected rat cardiac myocytes with c-myc–tagged dominant-negative (N17 or N19) or constitutively active (L61 or L63) mutants of Rho, and performed dual fluorescence microscopy for c-myc and intracellular oxidation. Treatment with AngII increased intracellular oxidation, as determined by DCF fluorescence in nontransfected cells or cells transfected with dominant-negative RhoA or Cdc42 (Figure 5a). Transfection with dominant-negative Rac1, however, completely inhibited AngII-induced intracellular oxidation. Conversely, only cells transfected with constitutively active Rac1 showed increased intracellular oxidation; nontransfected cells or cells transfected with constitutively active RhoA or Cdc42 showed no such increase (Figure 5b). These results indicate that Rac1 mediates AngII-induced intracellular oxidation, and suggest that Sim inhibits intracellular oxidation by inhibiting Rac1.

Statins inhibit cardiac hypertrophy in vivo. To determine whether our in vitro findings have physiological relevance, we evaluated the effects of statins using two widely used models of cardiac hypertrophy (35, 36). AngII infusion caused a substantial increase in systolic blood pressure that was not affected by cotreatment with Sim

(Table 1). Treatment with Sim alone had no effect on systemic blood pressure, BW, or total serum cholesterol levels. AngII infusion (200 ng/kg/day for 14 days) caused a significant increase in LV/BW ratio and LV mass. However, in the short duration of our experimental conditions, AngII did not produce significant deposition of collagen or fibrosis. The increases in LV/BW ratio and LV mass by AngII were inhibited in a concentration-dependent manner by cotreatment with Sim. Similar findings were also observed with atorvastatin, suggesting a class effect on myocardial HMG CoA reductase inhibition.

In hearts from rats treated with AngII infusion, there were increases in membrane-associated RhoA and Rac1 GTP-binding activities, both of which were inhibited by cotreatment with Sim (Figure 6a). Indeed, AngII increased $O_2\cdot^-$ production in intact, nonhomogenized rat heart tissues by 40% (from 42 ± 4 to 59 ± 5 nmol/mg,
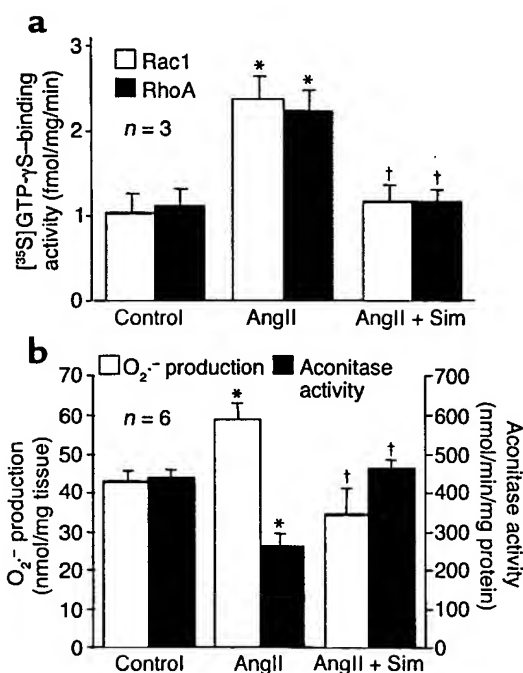
Figure 6

Inhibition of Rac1 and superoxide anion production in rat hearts. Effects of AngII (200 ng/kg/min) infusion with and without Sim (20 mg/kg/day for 14 days) on (a) membrane-associated Rac1 and RhoA GTP-binding activities and (b) aconitase activity and $O_2\cdot^-$ released from intact rat hearts. Values are expressed as mean ± SEM. *$P < 0.05$ compared with vehicle-treated animals (Control). †$P < 0.05$ compared with animals treated with AngII alone.

Table 1
Effects of statin on AngII-induced cardiac hypertrophy

Parameter	Control	AngII	AngII + Sim (0.2)	AngII + Sim (2)	AngII + Sim (20)	Sim (20)
Systolic BP (mmHg)	118 ± 8	180 ± 4*	177 ± 3*	178 ± 3*	173 ± 4*	119 ± 8
BW (g)	297 ± 6	291 ± 2	289 ± 6	285 ± 9	288 ± 5	287 ± 7
LV mass (mg)	642 ± 17	826 ± 23*	781 ± 16*	707 ± 26**†	679 ± 30†	624 ± 21†
LV/ BW (mg/g)	2.16 ± 0.03	2.84 ± 0.06*	2.71 ± 0.07*	2.49 ± 0.08**†	2.35 ± 0.10†	2.17 ± 0.05†
Serum cholesterol (mg/dl)	125 ± 9	131 ± 4	133 ± 8	129 ± 5	129 ± 5	126 ± 4

Sim is expressed in mg/kg/day. Values are mean ± SEM, n = 5–8 per group. *P < 0.01 versus control group. **P < 0.05 versus control group. †P < 0.01 versus AngII group. BP, blood pressure.

n = 6, P < 0.05), an effect that was completely blocked by cotreatment with Sim (34 ± 8 nmol/mg, n = 6, P > 0.05 compared to control) (Figure 6b). These results correlated inversely with a 41% decrease in aconitase activity in rat heart tissues after AngII infusion (from 440 ± 27 to 260 ± 41 nmol/min/mg, n = 6, P < 0.005), which was completely restored by cotreatment with Sim (460 ± 31 nmol/min/mg, n = 6, P > 0.05 compared to control). These findings suggest that inhibition of Rho proteins contributed to the statin's inhibitory effect on AngII-induced $O_2 \cdot^-$ production in vivo.

To determine whether the antihypertrophic effect of statins is a central mechanism that could be generalized to other etiologies of cardiac hypertrophy, we investigated the effects of Sim in a pressure-overload model of cardiac hypertrophy. TAC (aortic banding) did not affect systolic blood pressure or BW. However, TAC produced significant increases in the ratio of heart weight (HW) to BW compared with sham-operated mice (Figure 7a). These effects of TAC correlated with a 59% decrease in aconitase activity in mouse heart tissues (from 490 ± 22 to 201 ± 18 nmol/min/mg, n = 3, P < 0.01). Indeed, TAC resulted in anatomical and functional changes that were consistent with cardiac hypertrophy, such as increases in posterior wall thickness and LV end-systolic diameter, and a 25% decrease in fractional shortening or ejection fraction (Table 2). Cotreatment with Sim attenuated HW/BW ratio by approximately 50%, completely restored aconitase activity in mouse heart tissues, and improved cardiac diastolic and to a lesser extent, systolic function. Furthermore, treatment with Sim significantly decreased cardiac myocyte size, LV wall thickness, and gross cardiac dimensions (Figure 7b).

Figure 7
Inhibition of pressure overload–induced $O_2 \cdot^-$ production and cardiac hypertrophy by statins. (a) Effects of TAC with and without Sim (2 mg/kg/day for 4 weeks) on HW/BW ratio in mice and aconitase activity in mouse hearts. Values are expressed as mean ± SEM. *P < 0.01 compared with vehicle-treated sham-operated animals (Sham). †P < 0.05 compared with TAC treatment without Sim. (b) Cardiac hypertrophy was assessed by hematoxylin and eosin staining of heart tissues (top row; bar = 10 μm) and gross postmortem cross-sectional examination of the heart at the level just below the mitral valve (bottom row; bar = 1 mm).

Discussion
Statins prevent the development of cardiac hypertrophy in a cholesterol-independent manner. The mechanism is due in part to the inhibition of isoprenoid synthesis, Rho geranylgeranylation, and Rac1-induced $O_2 \cdot^-$ production in cardiac myocytes. Indeed, we found that the nonspecific antioxidant, N-acetylcysteine, was just as effective as statins in inhibiting AngII-induced cardiac hypertrophy (data not shown). However, further studies need to be performed to determine the precise mechanism by which N-acetylcysteine inhibits cardiac hypertrophy, and whether it involves its antioxidant effects. Nevertheless, the ability of statins to decrease $O_2 \cdot^-$ production via inhibition of Rac1 suggests that they may have clinical benefits beyond lipid lowering in attenuating the hypertrophic process.

Evidence is emerging that statins and antioxidants could have inhibitory effects on cardiac hypertrophy

a

b
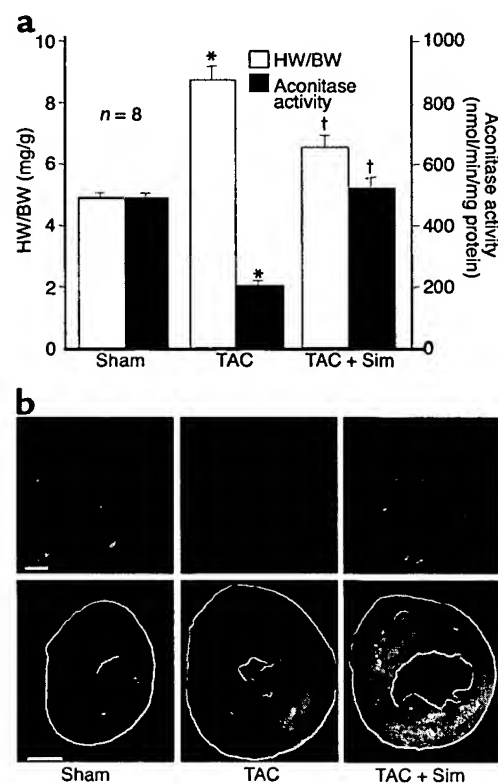
Table 2
Echocardiographic analysis of mice with TAC

Parameter	Sham	TAC	TAC + Sim
Systolic BP (mmHg)	117 ± 2	112 ± 2	114 ± 6
PWT, diastolic (mm)	0.69 ± 0.02	1.02 ± 0.03*	0.84 ± 0.06**†
LVD, diastolic (mm)	3.27 ± 0.17	3.44 ± 0.08	3.27 ± 0.17
LVD, systolic (mm)	1.95 ± 0.14	2.38 ± 0.10*	2.11 ± 0.13†
Fractional shortening (%)	41 ± 2	31 ± 2*	36 ± 2†

Values are mean ± SEM, $n = 8$ per group. *$P < 0.01$ versus sham group. **$P < 0.05$ versus sham group. †$P < 0.01$ versus TAC group. PWT, posterior wall thickness; LVD, left ventricular diameter.

(37–39), although the mechanism for these effects remains largely unknown. For example, a recent study showed that statins could inhibit cardiac hypertrophy and fibrosis in a rabbit β-myosin heavy chain-Q^{403} mutant model of cardiac hypertrophy without affecting Rac-GTP activity (38). These results suggest that the antihypertrophic effects of statins may not be due to the inhibition of Rho proteins alone, but may also involve the inhibition of other downstream signaling pathways. In contrast to other studies, we did not observe any increase in cardiac fibrosis in our AngII infusion model, probably due to the lower concentration of AngII and the shorter treatment period used in our study. Indeed, cardiac hypertrophy and fibrosis are observed with higher concentrations of AngII (40). Our findings are in agreement with a previous study showing that statins attenuate vascular smooth muscle cell hypertrophy by inhibiting Rac-induced $O_2\cdot^-$ production (32).

The antihypertrophic effect of statins could also be due, in part, to decreases in AngII type 1 receptor expression (32) or myocardial angiotensin-converting enzyme activity (37). However, the ability of statins to inhibit cardiac myocyte hypertrophy in response to other agonists such as endothelin-1 and phenylephrine, and to pressure overload, suggests that the predominant mechanism of action includes mechanisms that are distal to the AngII type 1 receptor. Statin therapy also increases vascular nitric oxide production (23), which can potentially decrease blood pressure and attenuate the hypertrophic process. However, there were no significant changes in systemic arterial systolic blood pressure in animals treated with statins in our study. Finally, nitric oxide may also contribute to the direct antihypertrophic effects of statins through favorable effects on cardiac performance and metabolism (41). Further studies with statins in eNOS$^{-/-}$ mice should help clarify these issues.

Statins inhibit the synthesis of important isoprenoid intermediates such as GGPP and FPP, which are important lipid attachments for the posttranslational modification of small GTP-binding proteins such as Rho (18, 19). Rho proteins play a critical role in mediating the development of cardiac hypertrophy. For example, RhoA controls the formation of actin stress fiber and focal adhesion complexes through activation of Rho kinases and myosin light chain phosphorylation (42).

Rac1 and Cdc42 regulate actin cytoskeletal processes called lamellipodia (43) and filopodia (44), which may be involved in the morphological changes associated with cardiac hypertrophy. In addition, Rho proteins could also regulate the hypertrophic process by activating downstream signaling molecules such as mitogen-activated protein (MAP) kinases (11, 13). Our finding that overexpression of dominant-negative Rac1 completely inhibited intracellular oxidation and cardiac myocyte hypertrophy, whereas RhoA caused a lesser inhibition and Cdc42 none at all, is in agreement with a previous study showing that Rac1 is required for signal transduction pathways leading to cardiac myocyte hypertrophy (15).

Since the hypertrophic response is a mechanism of physiological adaptation to increases in afterload, it is not entirely clear whether inhibition of the hypertrophic process at the expense of decreasing cardiac performance would actually be beneficial. For example, in experimental models of LV pressure overload, inhibition of the hypertrophic response leads to the rapid development of dilated cardiomyopathy and heart failure (45). Similar detrimental effects often occur following myocardial infarction, if the hypertrophic process or myocardial remodeling is inhibited (46). However, despite these concerns, our results indicate that statins not only decreased cardiac hypertrophy, but also improved cardiac performance by mostly reducing LV end-diastolic dimensions, even in the presence of persistent elevations in systemic blood pressure and afterload. The precise mechanism(s) underlying these properties of statins is unknown, but it may involve enhancing nitric oxide–mediated improvement in myocardial energy metabolism (41).

In summary, statins are effective agents for preventing the development of cardiac hypertrophy. Of clinical significance is that hypertension, not hypercholesterolemia, is the main risk factor for cardiac hypertrophy (2). These results, therefore, suggest a novel pharmacological approach to treating cardiac hypertrophy in a patient population where statin therapy may not otherwise be indicated. Supporting data from large clinical trials, however, are needed before such recommendations can be made.

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